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LOCALIZATION OF ADENOSINE 3′,5′-MONOPHOSPHATE IN MOUSE EPIDERMIS BY IMMUNOFLUORESCENCE

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Adenosine 3′,5′-monophosphate (cyclic AMP) has been localized in mouse epidermal cells using an immunofluorescent technique. Within 10 min following the intraperitoneal injection of isoproterenol or 30 hr following the topical application of croton oil in acetone, staining was clearly visible in the cytoplasm of the basal cells.

Epidermis, in common with other mammalian tissues, contains adenosine 3′,5′-monophosphate (cyclic AMP), and this material has been postulated to play an important role in the regulation of the growth and function of epidermal cells (for references, see [1]). Some work has been done also on the changes in epidermal cyclic AMP levels and on their responsiveness to hormones following the application of carcinogens and tumor promotors to mouse skin [2–4]. Although mouse epidermis is composed predominantly of keratinocytes, the cell population is a continuum varying from mitotically active cells in the basal layer, to dead, fully keratinized cells in the horny layer. Consequently, any attempt to correlate gross biochemical measurements of cyclic nucleotides or other components with, for example, the complex processes of tumor initiation and promotion will require information on the cytologic localization of these materials. Localization of cyclic nucleotides by the technique of fluorescence immunocytochemistry has been reported for a number of mammalian tissues [5–9]. In the present study, this technique has been applied to the localization of cyclic AMP in mouse epidermis after elevation of the basal levels of this nucleotide by injection with isoproterenol [2–4] or by the topical application of croton oil [4].

MATERIALS AND METHODS

Materials. Immunoglobulin fractions with a high binding capacity for cyclic AMP were prepared from antisera raised by repeated injections of rabbits with 2-0-succinyl cyclic AMP conjugated to human serum albumin [10]. Goat antibodies to rabbit IgG were obtained from Calbiochem Australia Ltd., Sydney, Australia, and fluorescein-labeled as described previously [11]. Croton oil and isoproterenol were obtained from the Sigma Chemical Co., St. Louis, Missouri.

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Histochemical procedure. Mice were kept and treated with croton oil or isoproterenol as described previously [4]. At appropriate times after treatment, animals were killed by cervical dislocation and the dorsal skin excised and immediately frozen in isopentane cooled by liquid nitrogen. Cryostat sections (6–8 μm in thickness) of unfixed tissue were transferred onto glass slides. The sections were treated in sequence with antibody to cyclic AMP, phosphate-buffered saline (pH 7.5), fluorescein-labeled antibodies to rabbit IgG, and phosphate-buffered saline (pH 7.5) as described earlier [5]. Finally, sections were mounted in 50% glycerin in phosphate-buffered saline and immediately examined by incident light–dark field fluorescence microscopy, using a HBO 100 mercury lamp, reflector F1 500, excitation filter KP 500, and interference barrier filter KP 630. Sections were photographed with Kodak Tri-X 35-mm in an automatic camera.

Measurement of cyclic AMP binding. The binding of [8-^{3}H]cyclic AMP to immunoglobulin fractions was measured by Millipore filtration as described previously [12], but with the omission of AMP from the assay mixtures. Control assays containing 6 pmoles of tritiated cyclic AMP and rabbit antibodies to cyclic AMP (103 μg of protein) bound 1.9 pmoles of cyclic AMP per mg of protein. This binding was not depressed by the inclusion of 50 or 250 pmoles of unlabeled AMP, ATP, or cyclic guanosine 3′,5′-monophosphate (cyclic GMP) in assay mixtures. The addition of 50 or 250 pmoles of unlabeled cyclic AMP decreased the binding of tritiated nucleotide by 78 and 94%, respectively.

No binding of tritiated cyclic AMP was detected in assays containing immunoglobulins purified from normal rabbit serum.

RESULTS

As shown in Figure 1, fluorescence indicating the presence of cyclic AMP was detected in epidermal cells following the injection of isoproterenol or the topical application of croton oil. Only weak staining was apparent in control animals injected with saline in place of isoproterenol (Fig. 1*B*), or treated with acetone in place of croton oil (data not shown). No fluorescence associated with epidermal cells was observed in sections treated with fluorescein-labeled goat antiserum to rabbit immunoglobulins but not treated with antibodies to cyclic AMP (Fig. 2*B*) or in unstained sections (data not shown). Essentially no staining was obtained using antibodies purified from normal rabbit serum (Fig.

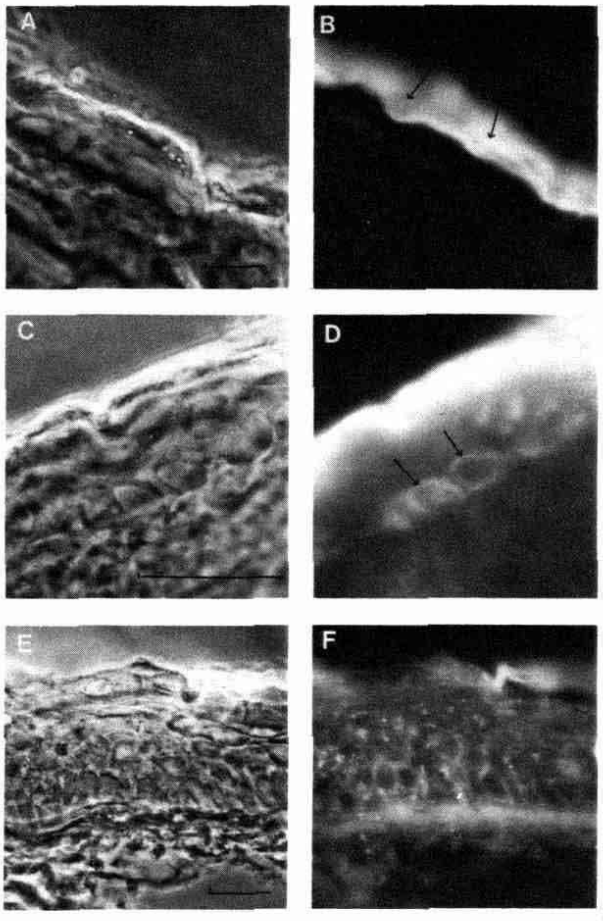

FIG. 1. Immunofluorescent localization of cyclic AMP in mouse epidermis. *A* (phase contrast) and *B* (fluorescence) are photomicrographs of a section prepared from a control mouse; *C* (phase contrast) and *D* (fluorescence) are photomicrographs of a section prepared 10 min after the injection of isoproterenol. *E* (phase contrast) and *F* (fluorescence) are photomicrographs of a section prepared 30 hr after the application of croton oil. *Arrows* point to areas of specific fluorescence. The bar corresponds to 50 μ.

2*D*) or following the prior incubation of the rabbit antiserum with 10^{-4} M cyclic AMP for 2 hr at 4°C (Fig. 2*F*). These results, together with the binding data reported in *Materials and Methods*, establish the specificity of the antibodies for cyclic AMP. In all of the control experiments summarized in Figure 2 a low level of nonspecific fluorescence was frequently observed in the dermal layer.

As is apparent in Figures 1 and 2, a strong autofluorescence was observed in the outer keratinized layer (e.g., see *arrows* in Fig. 1*B*). This autofluorescence was observed even in untreated slides mounted directly in glycerin (data not shown) and is therefore not related to the specific staining patterns obtained. In some cases the strength of this nonspecific fluorescence prevented sharp localization in photographs of specific staining in the epidermal cells (e.g., see Fig. 1*D*). The best localization of specific fluorescence was obtained when the keratinized layer was partly removed during section preparation (e.g., see Fig. 1*F*).

Isoproterenol injection induced about a 10-fold increase in epidermal cyclic AMP levels under the conditions used in these experiments [2–4]. This was associated with an increase in specific fluorescence, which was predominantly located in the cytoplasm of basal epidermal cells (Fig. 1*D*, see *arrows*). Although an occasional basal cell was observed which did not show fluorescent staining following isoproterenol injection, no evidence for a clustering of responsive cells was obtained. Croton oil treatment also induced specific cytoplasmic staining for cyclic AMP in the nucleated cells of the thickened epidermal layer (Fig. 1*E*, *F*). Some discrete areas of more intense staining were also repeatedly observed after croton oil treatment (see Fig. 1*F*), but it was often not possible to associate these with individual epidermal cells. A dispersed band of staining, also not obviously associated with

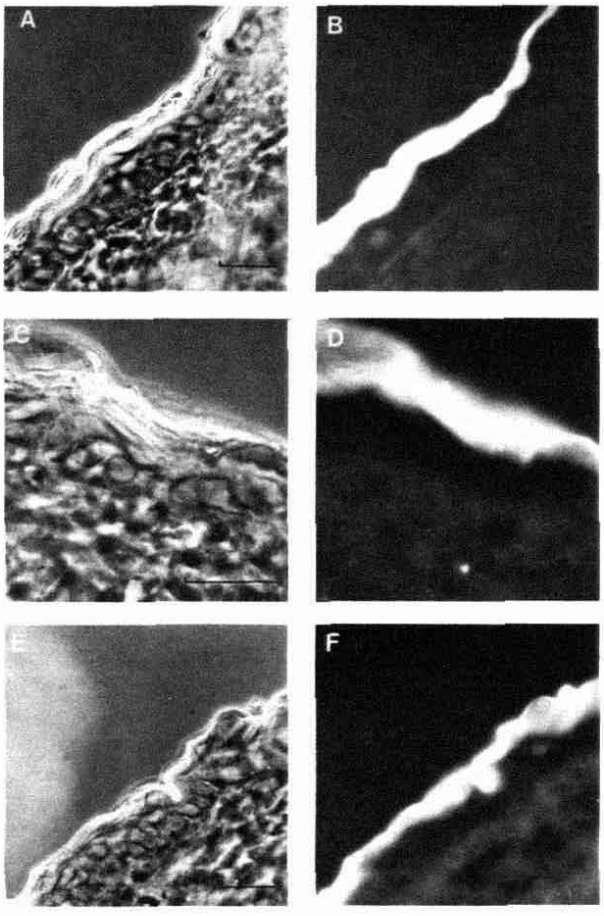

FIG. 2. Control experiments for the immunofluorescent localization of cyclic AMP in mouse epidermis. All sections represent separate experiments and were prepared 10 min after the injection of animals with isoproterenol. In *A* (phase contrast) and *B* (fluorescence) a section was treated with fluorescein-labeled goat antiserum to rabbit immunoglobulins but not with antibodies to cyclic AMP. In *C* (phase contrast) and *D* (fluorescence) a section was treated with fluorescein-labeled goat antiserum to rabbit immunoglobulins and with antibodies prepared from normal serum. In *E* (phase contrast) and *F* (fluorescence) a complete localization reaction was carried out, but the rabbit antiserum was preincubated with 10^{-4} M cyclic AMP (2 hr; 4°C). The bar corresponds to 50 μ.

particular epidermal cells, was apparent around the position of the basal lamina basement membrane.

DISCUSSION

Previous studies using keratotome slices [1] or epidermal scraping techniques [2–4] have indicated the presence of an adenylate cyclase responsive to β-receptor stimulators in rat, mouse, and human epidermis. The present results confirm directly the presence of cyclic AMP in mouse epidermal cells following stimulation with isoproterenol and suggest that the cyclic nucleotide is localized predominantly in the cytoplasm. A lack of intense staining for cyclic AMP in the cell nucleus has been observed in other tissues [6–9]. A different staining pattern has been observed for cyclic GMP in a number of tissues [6,8,9], and it will be of interest to extend this work to localization of epidermal cyclic GMP.

An essentially similar pattern of staining was observed in epidermal cells induced to accumulate cyclic AMP in response to application of the tumor promotor croton oil. A pronounced hyperplasia is evident 30 hr after the application of croton oil, and epidermal cyclic AMP levels are significantly increased [4]; the increase is sensitive to α-receptor blockers [3]. This is in direct contrast to the isoproterenol-stimulated increase in cyclic AMP, which is blocked by propanolol, a β-receptor antagonist [1,3,4]. Consequently, it was necessary to determine whether the cyclic AMP accumulated in response to both types of stimulation had a similar intercellular and intracellular localization. Despite a strong autofluorescence of the keratinized layer, a relatively sharp localization of specific staining was possible and it seems likely that the technique of immunocytochemistry will become a powerful tool in studies of the physiologic roles of cyclic nucleotides in complex tissues such as skin.

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